HISTOCHEMICAL STUDIES ON THE LIPID REQUIREMENT FOR THE OXIDATIVE ENZYME ACTIVITIES : II. STUDIES ON THE EFFECTS UPON THE OXIDATIVE ENZYME ACTIVITIES OF PHOSPHOLIPIDS, Co Q AND β-LIPOPROTEIN

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II. STUDIES ON THE EFFECTS UPON THE OXIDATIVE ENZYME ACTIVITIES OF PHOSPHOLIPIDS, Co Q AND β-LIPOPROTEIN

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INTRODUCTION

As has been described in the papers of the first part, it is evident that phospholipids participate specifically in the manifestation of histochemical activities of lactic dehydrogenase and DPNH diaphorase. The histological processes are essential for preserving valuable substances, such as phospholipids. Therefore, the kinds of organic solvent used in the histological processes as well as their duration of action, and the temperature of the paraffin are matters of great importance. To get an excellent histochemical result proper organic solvent should naturally be used throughout the whole process for preparing tissue sections. Here, several problems arise with reference to phospholipids. They are: (1) The relation between phospholipids and oxidative enzymes other than lactic dehydrogenase and DPNH diaphorase; (2) The relation between the oxidative enzymes and Co Q groups and β-lipoprotein; (3) The possibility of maintaining the activities of oxidative enzymes even after dewaxing by improving the techniques of removing paraffin from tissue sections fixed in chilled absolute acetone and embedded in soft paraffin; and (4) The expection that the diminished oxidative enzyme activity may be restored by the addition of phospholipids to the substrate mixtures.

The experiments described in this paper were done to clarify these problems. Also it was the author's wish to establish better methods for the histochemical demonstration of some oxidative enzymes and at the same time to elucidate the biological functions of phospholipids.

MATERIALS AND METHODS

Animal tissues used in the present experiments were obtained from the main organs of adult male albino rats of Wistar strain weighing from 140～160 g. Small tissue blocks ($1 \times 3 \times 3$ mm^3) obtained immediately after sacrifices were immersed in chilled absolute acetone for 6～9 hours at 3°C for dehydration and fixation, and then embedded in soft paraffin (melting point 48～50°C as described in previous paper). The thickness of the tissue sections used in the present experiments was 6～7μ, and absolute acetone was used for removing xylene in the dewaxing process.

Lipids used in the experiments were as follows: Four kinds of phospholipids, viz. lecithin, cephalin, phosphatidyl-serine and phosphatidyl-inositol. Lecithin, cephalin and phosphatidyl-serine were purchased from Tokyo Kasei Kogyo Co., Ltd. Phosphatidyl-inositol, Co Q_6 and Co Q_{10} were purchased from the Sigma Co., Ltd. In addition, as a special reagent β-lipoprotein was purchased from the California Corporation for Biochemical Research.

Phospholipids were homogenized in distilled water at a concentration of 0.5～1.0%. Co Q groups were dissolved in absolute acetone at a concentration of 0.1～0.5%. β-Lipoprotein, marketed as a 10% solution, was diluted to 0.5～1.0% solution with distilled water. Each of these prepared solutions was added to the substrate mixtures of the respective oxidative enzymes.

The enzymes observed with histochemical methods were as follows:

(1) Cytochrome oxidase
(2) Monoamine oxidase
(3) DPNH diaphorase
(4) TPNH diaphorase
(5) Succinic dehydrogenase
(6) Lactic dehydrogenase
(7) Malic dehydrogenase
(8) Iso-citric dehydrogenase
(9) β-Hydroxybutyric dehydrogenase
(10) Glutamic dehydrogenase
(11) Alcohol dehydrogenase
(12) Choline dehydrogenase
(13) β-Glycerophosphoric dehydrogenase
(14) 6-Phosphogluconic dehydrogenase
(15) Glucose-6-phosphoric dehydrogenase
(16) 3β-Hydroxysteroid dehydrogenase
(17) 17β-Hydroxysteroid dehydrogenase

(18) 17β-Oestradiol dehydrogenase
(18) Tetrahydrofolic dehydrogenase

The substrate mixtures for histochemical observations of the aforementioned oxidative enzymes were prepared as follows:

(1) The substrate mixtures for cytochrome oxidase

(a) Tetrazolium salt method[1),2),3)]

Sörenson's phosphate buffer (pH 7.4)	0.2 ml.
0.25 M p-Phenylenediamine	0.3 ml.
0.1% Nitro-blue tetrazolium	0.2 ml.
Distilled water	0.2 ml.
Lipid	0.05~0.3 ml.

(b) p-Aminodiphenylamine method[3),4),5),6)]

p-Aminodiphenylamine (base)	10~15 mg.
p-Methoxy-p-aminodiphenylamine	10~15 mg.
Ethanol	0.5 ml.

To the above-mentioned ethanol solution 35 ml of distilled water was added, and then 15 ml of 0.2 M tris buffer (pH 7.4) also was added. Following addition of buffer the solution was shaken and filtered through filter paper into a Coplin jar. To 3 ml of the prepared substrate mixture was added 0.15~0.9 ml of lipid solution.

(c) "Nadi" reagent method[3),7)]

Sörensen's phosphate buffer (pH 7.2~7.4)	25 ml.
1% α-Naphthol in 40% ethanol	2 ml.
1% N,N-dimethyl-p-phenylenediamine hydrochloride	2 ml.

To 3 ml of the prepared substrate mixture was added 0.15~0.9 ml of lipid solution.

(2) The substrate mixture for monoamine oxidase[3),8),9)]

Tryptamine hydrochloride	25 mg.
Sodium sulfate	4 mg.
0.1% Nitro-blue tetrazolium	2 ml.
M/10 Phosphate buffer (pH 7.6)	12 ml.

To 3 ml of the prepared substrate mixture was added 0.15~0.9 ml of lipid solution.

(3) The substrate mixture for DPNH diaphorase

DPNH	5~10 mg.
0.1% Nitro-blue tetrazolium	0.4 ml.
Sörensen's phosphate buffer (pH 7.4)	0.5 ml.
Lipid	0.05~0.3 ml.

(4) The substrate mixture for TPNH diaphorase

TPNH	5~10 mg.
0.1% Nitro-blue tetrazolium	0.4 ml.
Sörensen's phosphate buffer (pH 7.4)	0.5 ml.
Lipid	0.05~0.3 ml.

(5) The substrate mixture for succinic dehydrogenase

0.2 M Sod. succinate	0.35 ml.
0.1% Nitro-blue tetrazolium	0.20 ml.
Sörensen's phosphate buffer (pH 7.4)	0.35 ml.
0.1% Phenazine methosulfate	0.05~0.1 ml.
Lipid	0.05~0.3 ml.

For succinic dehydrogenase, each of the phospholipids also was added to substrate mixture with one of the Co Q group or β-lipoprotein at a time. When phospholipid was added to the substrate mixture with another lipid, the total amount to be added did not exceed 0.3 ml. at one time.

(6) The substrate mixture for lactic dehydrogenase

0.3 M Sod. lactate	0.4 ml.
0.1% Nitro-blue tetrazolium	0.3 ml.
Sörensen's phosphate buffer (pH 7.4)	0.4 ml.
DPN	5~10 mg.
Lipid	0.05~0.3 ml.

The pH of 0.3 M solution of sod. lactate was adjusted to 7.0 with NaOH.

(7) The substrate mixture for malic dehydrogenase

1.0 M Sod. malate	0.3 ml.
0.1% Nitro-blue tetrazolium	0.3 ml.
Sörensen's phosphate buffer (pH 7.4)	0.3 ml.
DPN	5~10 mg.
Lipid	0.05~0.3 ml.
Distilled water	0.2 ml.

The pH of 1.0 M solution of sod. malate was adjusted to 7.2 with 0.1 N NaOH solution.

(8) The substrate mixture for β-hydroxybutyric dehydrogenase

1.0 M Sod. β-hydroxybutyrate	0.3 ml.
0.1% Nitro-blue tetrazolium	0.3 ml.
Sörensen's phosphate buffer (pH 7.4)	0.4 ml.
DPN	5~10 mg.
Distilled water	0.2 ml.
Lipid	0.05~0.3 ml.

(9) The substrate mixture for glutamic dehydrogenase

1.0 M Sod. glutamate	0.3 ml.
0.1% Nitro-blue tetrazolium	0.3 ml.
Sörensen's phosphate buffer (pH 7.4)	0.4 ml.
DPN	5~10 mg.
Lipid	0.05~0.3 ml.

(10) The substrate mixture for iso-citric dehydrogenase

1.0 M iso-citric acid	0.30 ml.
0.1% Nitro-blue tetrazolium	0.30 ml.
Sörensen's phosphate buffer (pH 7.4)	0.40 ml.
Lipid	0.05~0.3 ml.
DPN	5~10 mg.

The pH of 1.0 M iso-citric acid solution was adjusted to 7.0 with Tris.

(11) The substrate mixture for alcohol dehydrogenase

1.0 M Ethanol	0.3 ml.
0.1% Nitro-blue tetrazolium	0.3 ml.
Sörensen's phosphate buffer (pH 7.4)	0.4 ml.
Lipid	0.05~0.3 ml.
DPN	5~10 mg.

(12) The substrate mixture for choline dehydrogenase

5% choline solution	0.3 ml.
0.1% Nitro-blue tetrazolium	0.3 ml.
DPN	5~10 mg.
Sörensen's phosphate buffer (pH 7.4)	0.4 ml.
Lipid	0.05~0.3 ml.

(13) The substrate mixture for α-glycerophosphate dehydrogenase

1.0 M α-glycerophosphate (disodium salt)	0.3 ml.
0.1% Nitro-blue tetrazolium	0.3 ml.
Tris-HCl buffer (pH 7.4)	0.4 ml.
DPN	5~10 mg.
Lipid	0.05~0.3 ml.

The pH of 1.0 M disodium α-glycerophosphate solution was adjusted to 7.2 with 0.1 N HCl.

(14) The substrate mixture for 6-phosphogluconic dehydrogenase

1.0 M Sod. 6-phosphogluconate	0.4 ml.
0.1% Nitro-blue tetrazolium	0.3 ml.
TPN	5~10 mg.
Tris-HCl buffer (pH 7.4)	0.4 ml.

Lipid	0.05～0.3 ml.

(15) The substrate mixture for glucose-6-phosphate dehydrogenase

1.0 M glucose-6-phosphate (disodium salt)	0.4 ml.
0.1% Nitro-blue tetrazolium	0.3 ml.
Tris-HCl buffer (pH 7.4)	0.4 ml.
TPN	5～10 mg.
Lipid	0.05～0.3 ml.

(16) The substrate mixture for 3β-hydroxysteroid dehydrogenase

Dehydroepiandrosterone, 10 mM in acetone	0.5 ml.
DPN, 1.3 mM in modified Krebs solution, pH 8.0	7.5 ml.
Nicotinamide, 40 mM in distilled water	1.0 ml.
Nitro-blue tetrazolium, 5 mg./ml. in distilled water	1.0 ml.

The modified Krebs solution consists of : 0.21 M NaCl, 1000 ml. ; 0.21 M KCl, 40 ml. ; 0.21 M $MgSO_4 \cdot 7H_2O$, 10 ml. ; 0.12 M phosphate buffer, 3000 ml. To 3 ml. of the prepared substrate mixture was added 0.15～0.9 ml. of solution.

(17) The substrate mixture for 17β-hydroxysteroid dehydrogenase

DPN	5～10 mg.
$MgCl_2$, 4.8 mg./ml. in distilled water	0.1 ml.
Nitro-blue tetrazolium, 65 mg./ml. disolved in N,N-dimethylformamide	0.1 ml.
Testosterone, 3.3 mg./ml. dissolved in N,N-dimethylformamide	0.1 ml.
Tris-HCl buffer (pH 8.4)	0.6 ml.
Distilled water	0.2 ml.
Lipid	0.05～0.3 ml.

(18) The substrate mixture for 17β-oestradiol dehydrogenase

DPN	5～10 mg.
$MgCl_2$, 4.8 mg./ml. in distilled water	0.1 ml.
Nitro-blue tetrazolium, 65 mg./ml. dissolved in N,N-dimethylformamide	0.1 ml.
17β-Oestradiol, 3.1 mg./ml. dissolved in N,N-dimethylformamide	0.1 ml.
Tris-HCl buffer (pH 8.4)	0.6 ml.
Distilled water	0.2 ml.
Lipid	0.05～0.3 ml.

(19) The substrate mixture for tetrahydrofolic dehydrogenase[10]

0.0025 M tetrahydrofolic acid	0.25 ml.
Tris-HCl buffer (pH 8.3)	0.40 ml.

0.1% aqueous solution of β-lipoprotein (bovine serum)	0.10～0.20 ml.
TPN	10 mg.
0.1% Nitro-blue tetrazolium	0.40 ml.
0.1 M $CaCl_2$ (or 0.1 M $BaCl_2$)	0.20 ml.
Distilled water	0.20 ml.
Lipid (other than β-lipoprotein)	0.05～0.3 ml.

For tetrahydrofolic dehydrogenase activity a test, in which β-lipoprotein was omitted from the substrate, also was done.

The incubation time for cytochrome oxidase activity ranged from 20 to 60 minutes in the tetrazolium method, and from 20 minutes to 24 hours both in the p-aminodiphenylamine method and the "nadi" reagent method. The incubation time for monoamine oxidase activity ranged from 30 minutes to 80 minutes. The incubation time for DPNH diaphorase and TPNH diaphorase activity ranged from 20 to 60 minutes. The incubation time for various other oxidative enzymes, such as succinic dehydrogenase and malic dehydrogenase, ranged from 20 minutes to 90 minutes.

RESULTS

There was recognized a general tendency for the enzyme activity to be slightly inhibited by excessive addition of phospholipids, Co Q group and β-lipoprotein to the substrate mixtures. The effects of each of the lipid reagents upon the enzyme activity are shown in table 1.

In the case of cytochrome oxidase observed by the tetrazolium method[1),2),3)] the enzyme activity was, of course, recognized positively in the tissue sections incubated in the substrate mixture to which 0.05～0.1 ml. of each of phosphlipids, Co Q group or β-lipoprotein was added. However, even though lipid solution was not added to the substrate mixture, the enzyme activity was positive. Moreover, on microscopic observation the histochemical findings in this case were much better than those obtained with substrate mixture containing lipid solution. The incubation time of 30～40 minutes was long enough to obtain good results with the tetrazolium method.

Control tests, using pre-treatment with KCN (10^{-2} M) and heating to 100°C for 20 minutes were almost negative.

On the other hand when tissue sections were incubated for 10～15 hours in each of the substrate mixtures of the p-aminodiphenylamine method[3),5),6)] and the "nadi" reagent method[3),7)], positive staining was recognized, but in these cases the staining was not inhibited at all by KCN pre-treatment and heating. Nor was the staining affected by the addition of any lipid solution.

Table 1. Effects of phosphatides, β-liprotein and $CoQ_{6,10}$ upon the histochemical reactions of oxidative enzymes.

Names of additions / Names of enzymes	Lecithin	Cephalin	Phosphatidyl-serine	Phosphatidyl-inositide	β-lipoprotein	CoQ_6	CoQ_{10}	No addition
Cytochrome oxidase	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Monoamine oxidase	(—)	(—)	(—)	(—)	(—)~(±)	(—)	(—)	(—)
NAD diaphorase	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(—)~(±)
NADP diaphorase	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(—)~(±)
Succinic dehydrogenase	(+)	(+)	(+)	(+)	(±)~(+)	(±)~(+)	(±)~(+)	(—)
Lactic dehydrogenase	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(—)
Malic dehydrogenase	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(—)
Iso-citric dehydrogenase	(±)~(+)	(±)~(+)	(—)~(±)	(±)~(+)	(±)~(+)	(—)~(±)	(—)~(±)	(—)
β-Hydroxybutyric dehydrogenase	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(±)~(+)
Glutamic dehydrogenase	(±)~(+)	(±)~(+)	(+)	(+)	(+)	(+)	(+)	(—)
Alcohol dehydrogenase	(±)~(+)	(±)~(+)	(±)	(±)	(±)	(±)	(±)	(—)
Choline dehydrogenase	(±)~(+)	(±)	(±)	(±)	(±)	(±)	(±)	(—)
α-glycerophosphoric dehydrogenase	(±)~(+)	(±)~(+)	(±)	(±)	(±)	(±)	(+)	(—)
6-phosphogluconic dehydrogenase	(—)	(—)	(—)	(—)	(—)	(—)	(—)	(—)
Glucose-6-phosphoric dehydrogenase	(—)	(—)	(—)	(—)	(—)	(—)	(—)	(—)
3β-Hydroxysteroid dehydrogenase	(—)	(—)	(—)	(—)	(—)	(—)	(—)	(—)
17β-Hydroxysteroid dehydrogenase	(—)	(—)	(—)	(—)	(—)	(—)	(—)	(—)
17β-oestradiol dehydrogenase	(—)	(—)	(—)	(—)	(—)	(—)	(—)	(—)
Tetrahydrofolic dehydrogenase	(—)	(—)	(—)	(—)	(+)	(—)	(—)	(—)

No difference was recognized in the activity of monoamine oxidase between the tissue sections incubated in the substrate mixture containing lipid and those incubated in the substrate mixture to which lipid was not added. The enzyme activity in both cases was almost negative, with only occasional faint staining.

The activities of DPNH diaphorase and TPNH diaphorase were very weak in the tissue sections incubated in the substrate mixture without lipid. However, when lipids were added to the substrate mixtures, the activities of the enzymes were markedly increased. There were no distinct differences in the degree of increase in the histochemical enzyme activity regardless of which lipid was added to the substrate mixtures.

The histochemical reaction of succinic dehydrogenase was almost negative in the tissue sections incubated in the substrate mixture of which lipid was not added. There were distinct differences in the succinic dehydrogenase activity depending on the kinds of lipids added to the substrate mixture. That is to say, the histochemical reaction of succinic dehydrogenase was remarkably positive in tissue section incubated in the substrate mixtures to which lecithin, cephalin, phosphatidyl-serine and phosphatidyl-inositol were added respectively. However, β-lipoprotein, Co Q_6 and Co Q_{10} could not sufficiently restore the activity of succinic dehydrogenase, and only faintly positive reactions were recognized. When any one of phospholipids, such as lecithin, cephalin, phosphatidyl-serine and phophatidyl-inositol, was added to the substrate mixture with Co Q_6, Co Q_{10}, or β-lipoprotein, good histochemical results were obtained.

In the cases of lactic dehydrogenase activity, histochemical reactions were very weak in tissue sections incubated in the substrate mixture containing no lipid. However, tissue sections incubated in the substrate mixture containing 0.05~0.1 ml. of lipid, the enzyme activity was markedly positive, showing clearly the restoration of enzyme activity. There was almost no difference in the degree of intensity of the lactic dehydrogenase activity in relation to the kinds of lipid added.

In the case of malic dehydrogenase activity, histochemical reactions were very weak in tissue sections incubated in the substrate mixture containing no lipid, and only faintly positive reactions were recognized. However, the addition of 0.05~0.1 ml. of lipid to the substrate mixture clearly restored the malic dehydrogenase activity, and the histochemical findings were excellent.

In the case of iso-citric dehydrogenase activity the histochemical enzyme reaction was almost negative in the absence of lipid, and on addition of lipid the degree of restoration of the enzyme activity was generally low. Particularly, the enzyme activity was not sufficiently restored by the addition of phosphatidyl-serine, Co Q_6 or Co Q_{10}.

The β-hydroxybutyric dehydrogenase showed moderately positive reactions even if none of the lipids was added. The enzyme activity was clearly increased by lipid addition. The addition of lecithin in particular gave excellent histochemical findings.

When none of the lipids was added to the substrate mixture, the histochemical reaction of glutamic dehydrogenase was almost negative. However, the addition of lipids restored the activity of the enzyme. The degree of

restoration of enzyme activity by lecithin or cephalin was somewhat weaker than that of the other lipids.

In the case of alcohol dehydrogenase activity the histochemical reaction was almost negative in the tissue sections incubated without lipids. Even in tissue sections incubated in lipid containing substrate mixture the restoration of the alcohol dehydrogenase activity was not sufficient. However, the degree of restortion of enzyme activity by lecithin or cephalin was a little better than with the others.

The histochemical reaction of the choline dehydrogenase was almost negative in tissue sections incubated without lipid. Though the activity of the enzyme was generally weak even in the case of lipid addition, with lecithin addition the histochemical findings were comparatively good.

In the case of α-glycerophosphate dehydrogenase the activity was almost negative in tissue sections incubated without lipid. However, when lecithin, cephalin or Co Q_{10} was added to the substrate mixture, the enzyme activity was sufficiently restored and the histochemical findings were comparatively good. None of the other lipids could restore sufficient activity and only slight positive reactions were seen.

In the cases of 6-phosphogluconic dehydrogenase, glucose-6-phosphate dehydrogenase, 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase and 17β-oestradid dehydrogenase the histochemical reactions were entirely negative with or without lipids.

In the case of tetrahydrofolic dehydrogenase[10] the activity was entirely negative without lipid, and only β-lipoprotein of the lipids tested was able to restore the enzyme activity.

As a result of the various experiments mentioned above, addition of phospholipids, Co Q group or β-lipoprotein to the substrate mixtures has made it possible to establish several new histochemical methods for certain oxidative enzymes.

The new methods will now be described:

(a) Histochemical method for the demonstration of cytochrome oxidase

(1) Dehydrate and fix a small fresh tissue block ($1\times3\times3\,mm^3$) for 6~9 hours in chilled absolute acetone (3°C) with several changes of the reagent in the initial 2~3 hours.

(2) Embed in soft paraffin (melting point 48~50°C) after clearing with xylene.

(3) Prepare tissue sections of 6~7μ thickness. Dewax with xylene, which should be removed by absolute acetone.

(4) After washing in distilled water for several minutes incubate tissue section at 37°C for 30~40 minutes in the following substrate mixture.

Sörensen's phosphate buffer (pH 7.4)	0.3 ml.
0.25 M p-phenylenediamine	0.3 ml.
0.1% Nitro-blue tetrazolium	0.2 ml.
Distilled water	0.2 ml.

(5) Wash sufficiently in running tap water.

(6) Dehydrate in graded alcohol series and mount in balsam after clearing in xylene. Or mount in glycerin or gum syrup.

For histochemical demonstration of the following enzymes to be described, the techniques of preparing tissue sections for incubation and the procedures for processing the tissue sections after incubation are the same as those for cytochrome oxidase. Therefore, only the composition of the substrate mixture and the incubation time for each of the enzymes will be described.

(b) Histochemical method for the demonstration of DPNH diaphorase

The substrate mixture

DPNH	5~10 mg.
0.1% Nitro-blue tetrazolium	0.4 ml.
Sörensen's phosphate buffer (pH 7.4)	0.5 ml.
Lecithin (cephalin, phosphatidyl-serine, phosphatidyl-inositol, Co Q_{16}, Co Q_{10} or β-lipoprotein)	0.05~0.1 ml.
Distilled water	0.2 ml.

The concentration of phospholipid solution is 0.5~1.0%. Co Q_6 and Co Q_{10} are dissolved in absolute acetone at a concentration of 0.1~0.5%, β-Lipoprotein was diluted to 0.5~1.0% solution. As the concetration of lipid solution is the same as above it will be omitted in thd following description. The incubation time is from 40~60 minutes.

(c) Histochemical method for the demonstration of TPNH diaphorase

The substrate mixture

TPNH	5~10 mg.
0.1% Nitro-blue tetrazolinm	0.4 ml.
Sörensen's phosphate buffer (pH 7.4)	0.5 ml.
Lecithin (cephalin, phosphatidyl-serine, phosphatidyl-inositol, Co Q_6, Co Q_{10} or β-lipoprotein)	0.05~0.1 ml.
Distilled water	0.2 ml.

The incubation time is 40~60 minutes.

(d) Histochemical method for the demonstration of succinic dehydrogenase

The substrate mixture

0.2 M Sod. succinate	0.4 ml.
0.1% Nitro-blue tetrazolium	0.3 ml.
Sörensen's phosphate buffer (pH 7.4)	0.4 ml.
0.1% Phenazine methosulfate	0.05～0.1 ml.
Lecithin (cephalin, phosphatidyl-serine or phosphatidyl-inositol)	0.05～0.1 ml.
Co Q_6 (or Co Q_{10})	0.05～0.1 ml.

The incubation time is 30～50 minutes.

(e) Histochemical method for the demonstration of lactic dehydrogenase

The substrate mixture

0.3 M Sod. lactate	0.4 ml.
0.1% Nitro-blue tetrazolium	0.3 ml.
Sörensen's phosphate buffer (pH 7.4)	0.4 ml.
DPN	5～10 mg.
Lecithin (cephalin, phosphatidyl-serine, phosphatidyl-inositol, Co Q_6, Co Q_{10} or β-lipoprotein)	0.05～0.1 ml.

The pH of 0.3 M sod. lactate solution is adjusted to 7.0 with sod. hydroxide.

The incubation time is 40～60 minutes.

(f) Histochemical method for the demonstration of malic dehydrogenase

The substrate mixture

1.0 M Sod. malate	0.3 ml.
0.1% Nitro-blue tetrazolium	0.3 ml.
Sörensen's phosphate buffer (pH 7.4)	0.3 ml.
DPN	5～10 mg.
Lecithin (cephalin, phosphatidyl-serine, phosphatidyl-inositol, β-lipoprotein, Co Q_6 or Co Q_{10})	0.05～0.1 ml.
Distilled water	0.2 ml.

The pH of 1.0 M sod. malate solution is adjusted to 7.2 with 0.1 N NaOH.

The incubation time is 30～50 minutes.

(g) Histochemical method for the demonstration of β-hydroxybutyric dehydrogenase

The substrate mixture

1.0 M Sod. β-hydroxybutyrate	0.3 ml.
0.1% Nitro-blue tetrazolium	0.3 ml.
Sörensen's phosphate buffer (pH 7.4)	0.4 ml.
DPN	5～10 mg.
Distilled water	0.2 ml.

Lecithin	0.05～0.1 ml.

The incubation time is 40～60 minutes.

(h) Histochemical method for the demonstration of glutamic dehydrogenase

The substrate mixture

1.0 M Sod. glutamate	0.3 ml.
0.1% Nitro-blue tetrazolium	0.3 ml.
Sörensen's phosphate buffer (pH 7.4)	0.4 ml.
DPN	5～10 mg.
Lecithin ($Co\,Q_6$, $Co\,Q_{10}$, β-lipoprotein, cephalin, phosphatidyl-serine or phosphatidyl-inositol)	0.05～0.1 ml.

The incubation time is 40～60 minutes.

It was not possible to establish a practical improved histochemical method for each of the following dehydrogenases; iso-citric dehydrogenase, alcohol dehydrogenase, choline dehydrogenase, α-glycerophosphate dehydrogenase, 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, 17β-oestradiol dehydrogenase and monoamine oxidase.

DISCUSSION

As has been clearly demonstrated by the experiments described in the paper of Part 1 the cause of reduction or disappearance of activities of some oxidative enzymes after dewaxing is the loss of phospholipids from the cells by alcohol extraction. Therefore, it is necessary to use in the histological processes an organic solvent which does not cause denaturation of enzyme protein nor extract any important chemical substances, such as phospholipids. In this respect it is very important to use absolute acetone throughout the whole process of preparation of tissue sections, because it has been generally said that absolute acetone does not dissolve phospholipids nor denature enzyme protein. However, according to the experiments of Shapiro *et al.* absolute acetone also is able to dissolve a considerable amount of phospholipids in the cells. The results of the present experiments are consistent with those of Shapiro *et al.*[11),12)]. That is to say, even if absolute acetone is used throughout the whole procedure for preparing tissue sections the activities of some oxidative enzymes are still considerably reduced or almost lost. As has been demonstrated already in the paper of Part 1 this fact can be reasonably interpreted to be due to the extraction of a considerable amount of phospholipids by absolute acetone. Therefore, further improvement will be necessary on the histological procedures for preparing tissue sections.

Since the activities of some oxidative enzymes is considerably reduced

even by absolute acetone, a solution of phospholipids, Co Q group or β-lipoprotein is used to restore the reduced enzyme activities.

It has been reported that when cytochrome oxidase extracted and purified in crystaline form was processed with methanol, lecithin was mainly extracted, and on the other hand catalytic activity of the enzyme disappeared, but that the enzyme activity was restored by the addition of extracted lecithin to the enzyme protein[13),14)]. It also has been reported that oxidative enzyme activity, such as DPNH-cytochrome c-reductase was greatly reduced or had almost disappeared with loss of phospholipids in the course of preparation of enzyme from mitochondria using deoxycholate or organic solvents, but the reduced enzyme activity was restored by the addition of phospholipids to the enzyme protein[15),16),17)]. However, in the case of cytochrome oxidase the result of the present histochemical experiments does not coincide with those of the above-mentioned biochemical investigations. This inconsistency, which also suggests the sites of enzyme localisation, may be resonably elucidated as follows: Namely, according to the investigations of D. E. Green[15)], it is suggested that cytochrome oxidase occupies the area of the neck of the structures protruding into the inner space of mitochondria. If, as Green claims, the cytochrome oxidase localizes at areas of the mitochondrial structure where influences can not easily extend from outside, it may reasonably speculated that the sites of cytochrome oxidase localization are almost unaffected by absolute acetone in case of tissue sections of 6~7μ thickness for the histchemical demonstration of cytochrome oxidase. Therefore, phospholipids at the areas of cytochrome oxidase may be preserved in relatively good condition. For the reason mentioned above, it may not be necessary to incorporate any extra phospholipids into the substrate mixture for the histochemical demonstration of cytochrome oxidase.

As for succinic dehydrogenase, β-hydroxybutyric dehydrogenase, and some other dehydrogenases it has been reported that the activity of these oxidative enzymes also will disappear with the loss of phospholipids[15),16),17),18),19),20),21)].

From the present experiments no definite comments can be made about the relationship between the activity of oxidative enzymes, (other than succinic dehydrogenase), and the Co Q group, in spite of an increase in some oxidative enzyme activity as mentioned above.

As in the case of tetrahydrofolic dehydrogenase, β-lipoprotein may be the best reagent for the restoration of reduced activity of oxidative enzymes, because it must contain several kinds of phospholipids in suitable hydrophilic form. In general the following inclination was recognized in the present

experiments; the oxidative enzymes in which reduced activities were restored by the addition of phospholipids and so on have been reported to be mainly localized in mitochondria, while in those reported to be independent of mitochondria the activities could not be restored. However, monoamine oxidase and lactic dehydrogenase are exceptions. Further histochemical studies should be performed on the relationship between the activity of the two enzymes and cellular lipids. The lipid requirement of some oxidative enzymes is very significant, in that it generally suggests enzyme localizations in the intracellular ultrastructures.

Since the non-specific reduction of tetrazolium salt[10),11),22),23)] which is a disadvantageous reaction in histochemistry of dehydrogenases in fresh and unfixed tissue sections was not recognized in the tissue sections of 6~7μ thickness after dewaxing even at high alkaline pH (about 9.3), the new improved histochemical methods are much better than the methods using cryostat tissue sections.

SUMMARY

(1) It has been made clear that the loss of phospholipids from cells by alcohol or some of the other organic solvents used in the histological procedures generally causes the reduction or disappearance of the activities of some oxidative enzymes, such as DPNH diaphorase, TPNH diaphorase, succinic dehydrogenase, lactic dehydrogenase, malic dehydrogenase, β-hydroxybutyric dehydrogenase and other oxidative enzymes that are in close relation to mitochondria, and that the reduced or lost activities of these oxidative enzymes can be restored by phospholipids, Co Q group or β-lipoprotein.

(2) The lipid requirement of a group of the oxidative enzymes proved to be closely connected with the ultrastructural localization of the enzymes.

(3) On the basis of the lipid requirement of the oxidative enzymes new improved methods have been established for the histochemical demonstrations of cytochrome oxidase, DPNH diaphorase, TPNH diaphorase, succinic dehydrogenase, lactic dehydrogenase, malic dehydrogenase, β-hydroxybutyric dehydrogenase and glutamic dehydrogenase.

(4) In these new methods the non-specific reduction of tetrazolium salt was not seen even at high alkaline pH (about 9.3). These new histochemical methods are thus much better than the methods in use at present.

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Explanation of Microphotographs

(1) Skeletal muscle : 100×

Cytochrome oxidase in tissue section which was fixed in absolute acetone and embedded in soft paraffin. Absolute acetone was used for removing xylene in dewaxing process. Two kinds of muscle fibres are seen in this picture; the first with relatively weak emzyme activity and the second with strong activity. It is evident in both muscle fibres that the enzyme is localized in mitachondria-like intracellular fine structures. The modified tetrazolium method.

(2) Liver : 100×

Cytochrome oxidase. The strong activity is localized in the cytoplasms of hepatic cells. However, no nuclear staining is seen. The modified tetrazolium method.

(3) Heart : 100×

Cytochrome oxidase. The enzyme activity is localized in the cytoplasms and no nuclear staining is seen. It is evident that the enzyme activity is localized in mitochondria-like intracellular fine structures. There are two kinds of muscle fibres; the first with relatively weak activity and the second with strong activity. The modified tetrazolium method.

(4) Testicle : 100×

Succinic dehydrogenase activity. Strong positive reaction is seen in the epithelial cells of seminiferous tubules, spermatozoa and basement membrane. Especially, the reaction of spermatozoa is intense. In this case tissue section was incubated in the substrate mixture containing lecithin.

(5) Liver : 100×

Succinic dehydrogenase activity in tissue section incubated in the substrate mixture containing lecithin. The enzyme activity is strong in the cytoplasms of hepatic cells situated at the central parts of the lobules. No nuclear staining is seen. It is evident that the enzyme activity is localized in mitochondria-like intracellular fine structures.

(6) Stomach : 100×

DPNH diaphorase activity in tissue section incubated in the substrate mixture containing phosphatidyl-serime. Strong activity of the enzyme is seen in the cytoplasms of chief cells and weak reaction is seen in parietal cells. No. nuclear staining is seen.

(7) Lung : 100×

TPNH diaphorase activity in tissue section incubated in the substrate mixture containing phosphatidyl-serine. Strong positive reaction is seen only in the epithelial cells of bronchus.

(8) Stomach : 100×

TPNH diaphorase activity in tissue section incubated in the substratae mixture containing phosphatidyl-serine. Strong positive reaction is seen in the cytoplasms of chief cells. In the muscular layer positive staining is seen. However, no nuclear staining is seen.

(9) Heart : 100×

TPNH diaphorase activity in tissue section incubated in the substrate mixture containing phosphatidyl-serine. There are two kinds of muscle fibres; the first with relatively weak activity and the second with strong activity. In both muscle fibres no nuclear staining is seen. It is evident in this picture that the activity is localized in mitochondria-like intracellular fine structures.

(10) Stomach : 100×

Lactic dehydrogenase activity in tissue section incubated in the substrate mixture containing phosphatidyl-serine. Strong positive reaction is seen in the cytoplasms of chief cells. Weak reaction is seen in muscular layer. However, no nuclear staining is seen.

(11) Kidney : 100×

Lactic dehydrogenase activity in tissue section incubated in the substrate mixture containing phosphatidyl-serine. Strong positive reaction is recognized in the epithelial cells of proximal convolutions and loops of Henle. However, no positive reaction is seen in glomeruli and basement membrane.

(12) Stomach : 100×

Glutamic dehydrogenase activity in tissue section incubated in the substrate mixture

containing phosphatidyl-serine. Strong positive reaction is seen in the cytoplasms of chief cells. No nuclear staining is recognized. Weak positive reaction is seen in muscular layer.

(13) Liver : 100×

Glutamic dehydrogenase activity in tissue section incubated in the substrate mixture containing β-lipoprotein. Positive staining is seen in the cytoplasms of hepatic cells. In general, however, the enzyme activity is stronger in the cells situated at the central parts of the lobules than in those situated at the peripheral parts. No nuclear staining is recognized. It is evident from this picture that the enzyme activity is localized in mitochondria-like intracellular fine structures.

(14) Kidney : 100×

Glutamic dehydrogenase activity in tissue section incubated in the substrate mixture containing β-lipoprotein. Strong positive reaction is recognized in the epithelial cells of proximal convolutions and loops of Henle. No nuclear staining is seen. No positive reaction is recognized in glomeruli and basement membrane.

(15) Liver : 100×

Glutamic dehydrogenase activity in tissue section incubated in the substrate mixture containing Co Q_{10}. Strong positive reaction is seen in the cytoplasms of hepatic cells. No nuclear staining is seen.

(16) Liver : 100×

Iso-citric dehydrogenase activity in tissue section incubated in the substrate mixture containing lecithin. Strong positive reaction is seen in the cytoplasms of hepatic cells. No nuclear staining is seen. The enzyme activity is assumed to be localized in mitochondria-like intracellular fine structures.

(17) Liver : 200×

Higher magnification of the previous picture. From this picture it is evident that the enzyme activity is localized in mitochondria-like intracellular fine structures. No nuclear staining is seen.

(18) Stomach : 100×

Iso-citric dehydrogenase activity in tissue section incubated in the substrate mixture containing lecithin. Strong positive reaction is recognized in the cytoplasms of chief cells. Moderate reaction is seen in muscular layer.

(19) Heart : 100×

Iso-citric dehydrogenase activity in tissue section incubated in the substrate mixture containing lecithin. There are recognized two kinds of muscle fibres; the first with moderate activity and the second with strong activity. No nuclear staining is seen.

(20) Liver : 100×

Malic dehydrogenase activity in tissue section incubated in the substrate mixture containing β-lipoprotein. Positive reaction is seen in the cytoplasms of hepatic cells. From the picture it will be assumed that the activity of the enzyme is localized in mitochondria-like intracellular fine structure. The activity is generally stronger in the central zone of the hepatic lobules than in the peripheral zone.

(21) Pancreas : 100×

Malic dehydrogenase activity in tissue section incubated in the substrate mixture containing β-lipoprotein. Moderate positive reaction is seen in the cytoplasms of pancreatic cells.

(22) Kidney : 100×

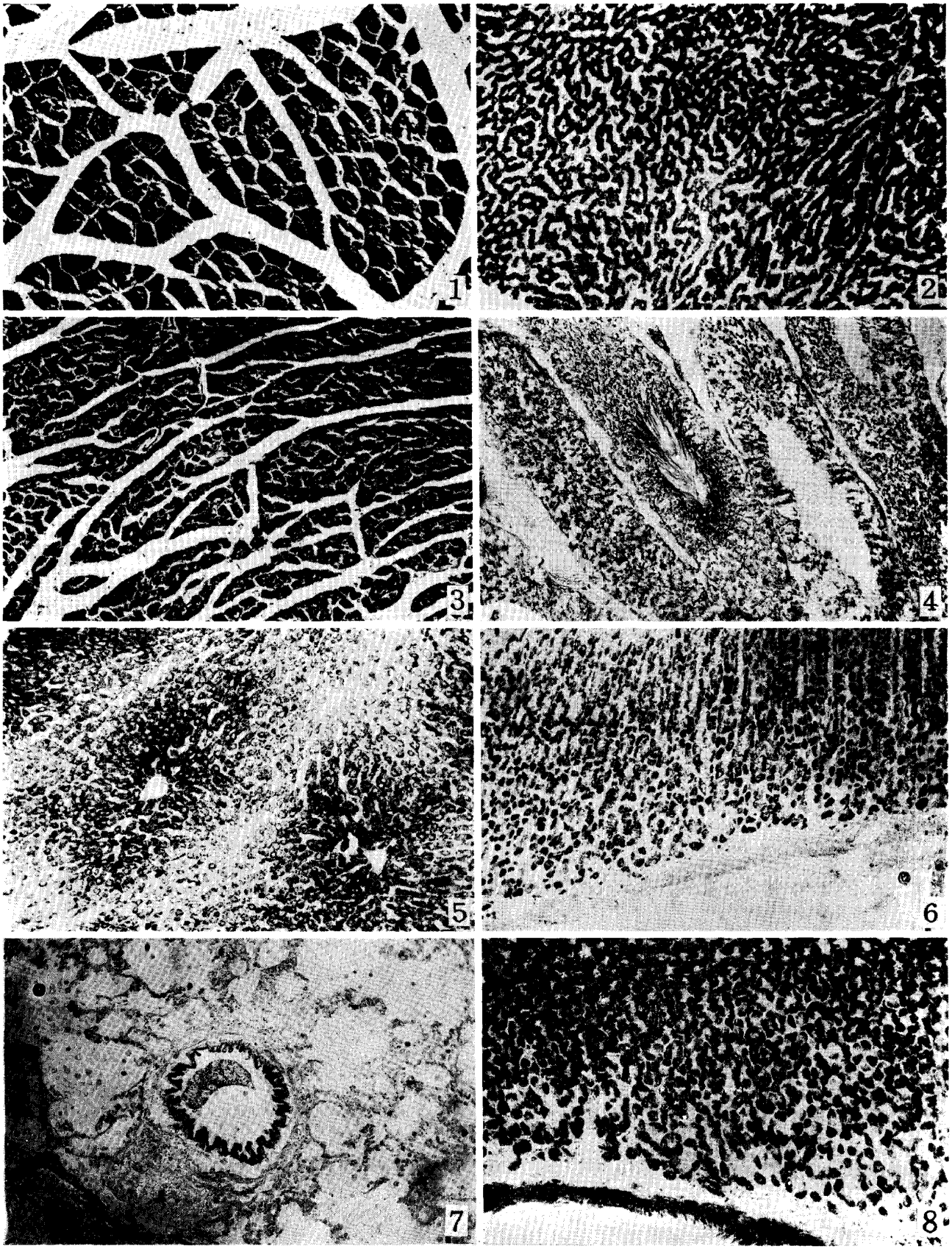
Malic dehydrogenase activity in tissue section incubated in the substrate mixture containing β-lipoprotein. Strong positive reaction is seen in the cytoplasms of the epithelial cells of proximal convolutions and loops of Henle. No staining is seen in nuclei and glomeruli.

(23) Liver : 200×

Malic dehydrogenase activity in tissue section incubated in the substrate mixture containing Co Q_{10}. Strong activity of the enzyme is seen in the cytoplasms of the hepatic cell. From this picture it is reasonably assumed that the activity of the enzyme is localized in mitochondria-like intracellular fine structure.

(24) Kidney : 100×

Malic dehydrogenase activity in tissue section incubated in the substrate mixture containing Co Q_{10}. Strong positive reaction is recognized in the cytoplasms of the epithelial cells of proximal convolutions and loops of Henle. No staining is recognized in nuclei and glomeruli.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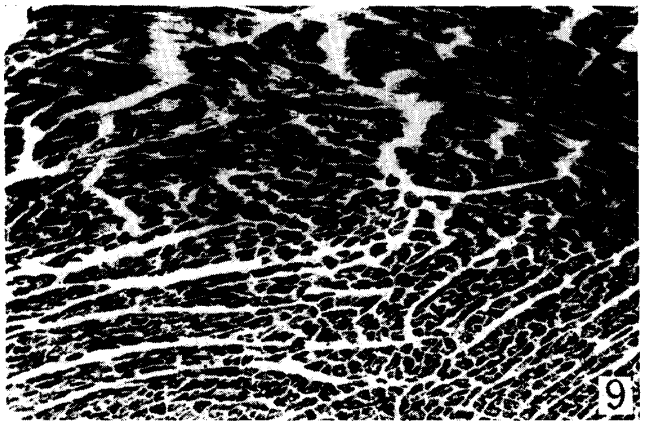
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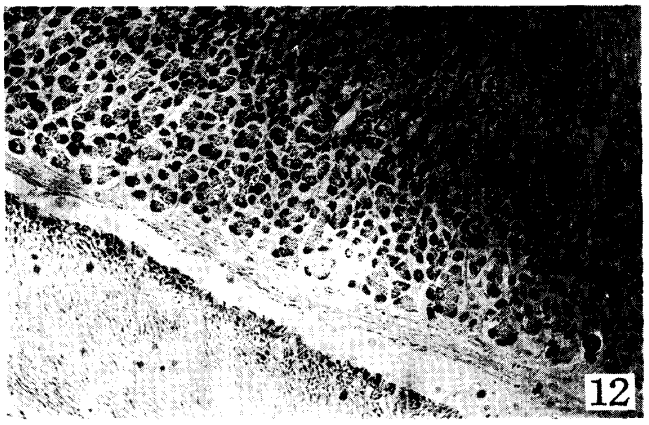
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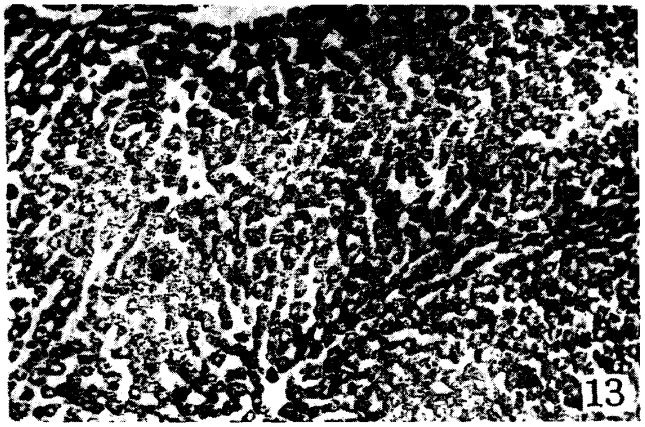
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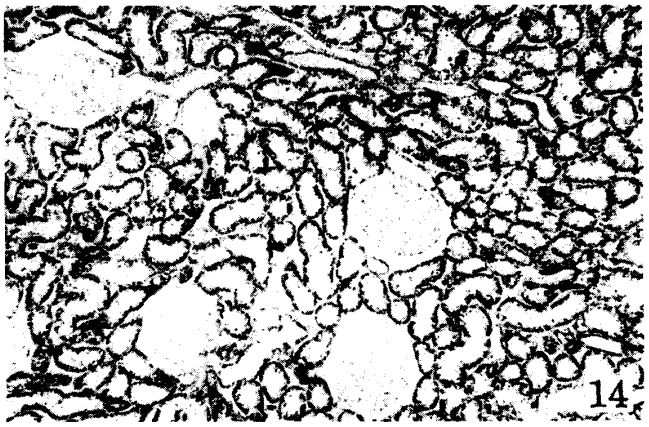
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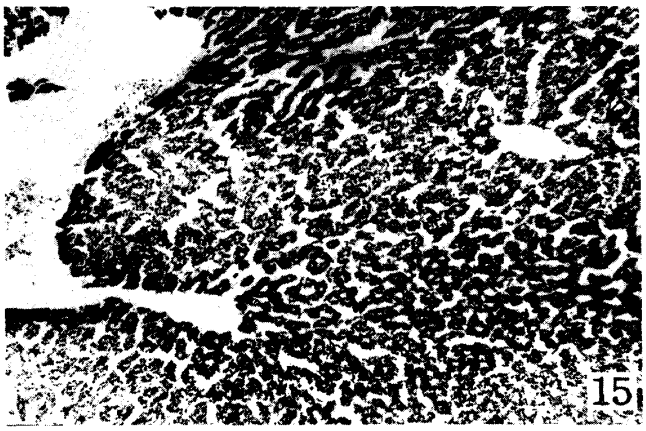
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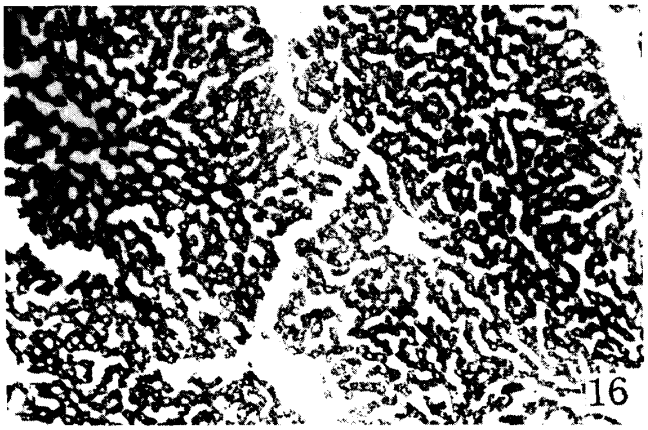
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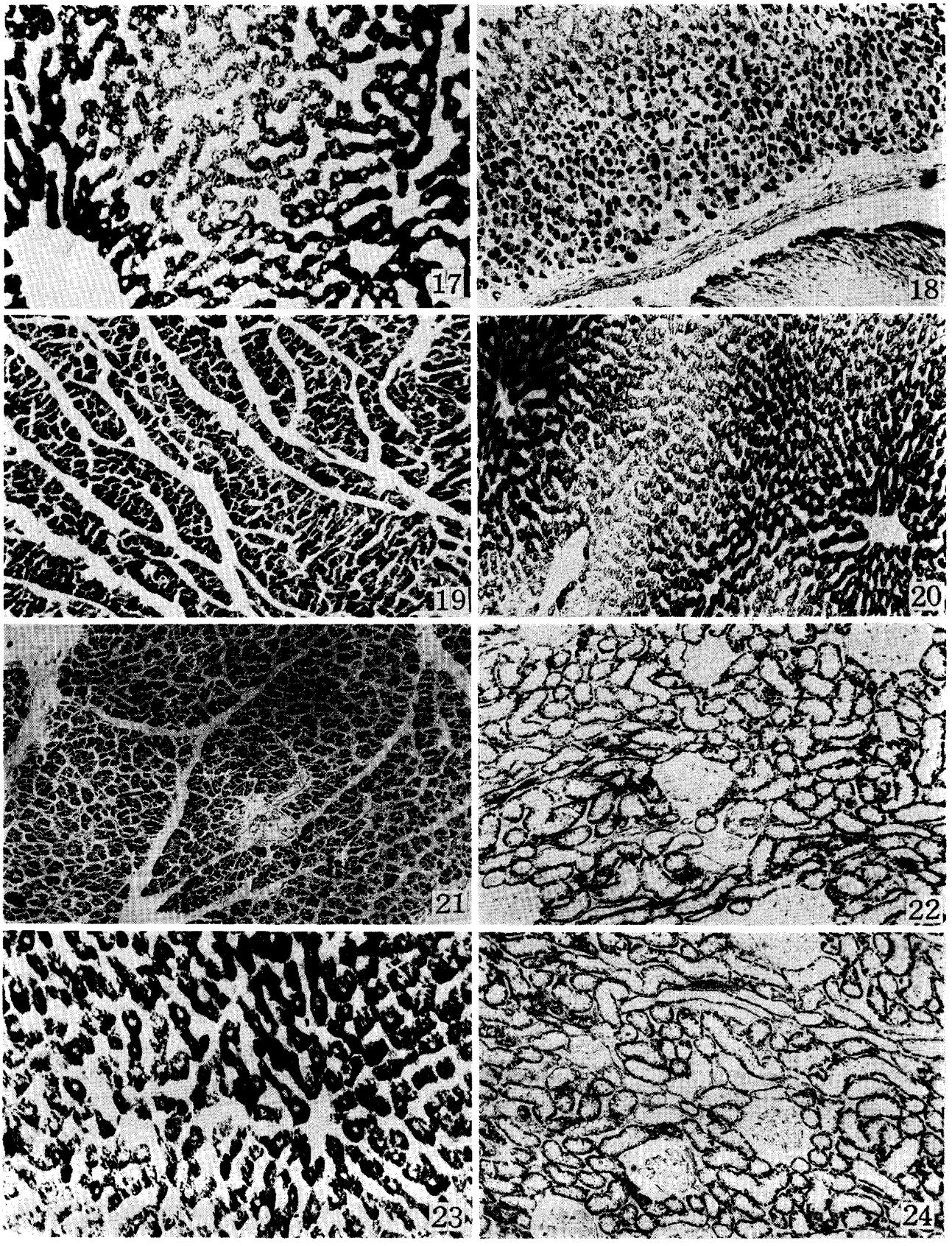
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